

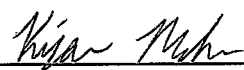
Characterizing the Antibacterial Properties of *Chimaphila maculata*.

Senior Project

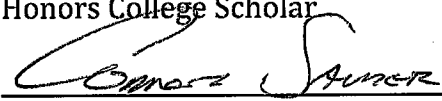
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The Esther G. Maynor Honors College
University of North Carolina at Pembroke

By

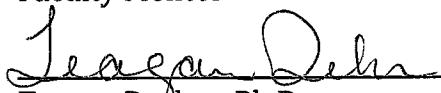
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Acknowledgements

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Abstract

Increasingly we are understanding that metabolic disorders, such as diabetes, involve disruptions in the pattern of microbial organisms, or microbiome, living within us. Addressing the disruption in microbiota is therefore a possible therapeutic avenue to treat these disorders. The overall goal of this project is to characterize the antimicrobial properties of plant-based teas used in traditional medicine to treat diabetes. *Chimaphila maculata*, commonly known as both pipsissewa and spotted wintergreen, is a plant frequently used by Southeastern American Indian communities as a treatment for digestive and metabolic disorders. The leaves, stems and roots of *C. maculata* plant were collected from Sampson's Landing in Pembroke, NC. Water-based extracts (teas) were created using these plant parts to use in agar diffusion assays. Briefly, to test for antimicrobial properties, filter discs soaked in teas were applied to agar plates smeared with 12 laboratory strains of human-dwelling bacteria. Teas with concentration ranging from 28% to 80% inhibited the growth of *S. aureus*, *B. subtilis*, *P. mirabilis*, *P. vulgaris*, *C. xerosis* and *E. faecalis*. These results suggest *C. maculata* teas used in traditional medicine may have antibacterial properties and therefore, may provide an alternative approach to treating disrupted microbiomes in diabetes.

Introduction

Before the discovery modern medicines and drugs that are used to treat diseases, there was traditional medicine (also known as herbal medicine). The definition of herbal medicine as defined by the World Health Organization is a medicinal product that contains some portion of plant material (Parveen, 2015). Indigenous people use and continue to use the plants of the Earth in order to treat diseases and illness. Studying the different properties of the plants allowed them to figure out which plants could be used to treat certain diseases. Sometimes these findings were documented, other times there were spread by word of mouth. Although there is not a great deal of research in this field, the studies on this topic shows that many of these plants and herbs were effective in treating disease. In most cases, however, we do not know the mechanism of how the plant impacts the body.

Over time, western medicine became the most common way to treat diseases and illness and traditional medicines were used less frequently. Although the use of western medicine has proven to be effective, there are many times complications and side effects that follow the uses of these medicines. According to Kooti (2016), herbal medicine is affordable and may have less side effects than drugs. Reintroducing the use of traditional medicine into our current health care methods could be a new way to treat disease while decreasing the amount of side effects that occur.

The importance of understanding traditional medicine are numerous. One main reason is that people are using traditional medicine and all of the side effects from this treatment has not been studied. People perceive herbal medicine as being safer because there are no chemicals but the reality is that these remedies can be just as dangerous. These plants can cause allergic reactions, toxicity in the body and may interact with other herbs or drugs (Meamarbashi, 2017). It has been proven that these plants can help with sickness but there is not a lot of scientific study

about how the plants are actually healing the body. The plants could heal a person's stomach ache but if it is also destroying healthy gut bacteria, then this is something that needs to be known before more harm than good is caused for that person. Also if the healing properties of the plant interact with other medicine that a person is taking, this could cause harm to someone. Both medicinal treatments could cancel each other out and the person would be receiving no treatment at all. Also the plant and other medicinal treatment could react dangerously with each other and cause harm to the person using these treatments together. For the health and safety of the people using these treatment options, it is important to study this topic more.

Another reason why this topic needs to be studied is because herbal medicine has been shown to be more convenient and accessible to people who may not be able to pay for expensive medications. A huge percentage of Africans use some form of traditional medicine (Tilburt, 2008). If a person is able to grow or collect plants, then there is a possibility for them to have easier access to these plants than to a pharmacy. Some of the herbs and plants can be grown in a garden and whenever that person needs to get some to make a tea or a salve then all they have to do is step outside and get some. Not everyone in the world has health insurance and access to a pharmacy so traditional medicine could be a solution to some global health issues (Tilburt, 2008).

There are a few studies about how herbal medicine has been used to treat diabetes and symptoms associated with it. One problem that is prevalent in diabetic patients is high glucose levels and managing those glucose levels. One study using herbal medicine in diabetes treatment used *Centella asiatica* extract. This herb was found to "reverse the glucose and lipid levels, as well as the tricarboxylic acid cycle and amino acid metabolic disorders back towards normal states" (M., Abas, 2016). This extract was also found to increase the production of insulin. Another study used *Urtica dioica* extract along with swimming to treat diabetes in lab mice. The result of using this herb in the study was a decrease in the serum glucose concentration, an increase in the resistance and sensitivity of insulin, a regeneration

of Langerhans islets with less beta cell damage and an increase in the secretion of insulin and glucose uptake (Ranjbari, 2016). Another problem for individuals with type 2 diabetes is the level of glucose uptake in the muscles. A study used *Tinospora crispa* as the treatment herb for glucose uptake problems. This herb stimulated insulin secretion in cells and caused enhancement of time and dose-dependent glucose uptake in muscles (Thomas, 2016). These are just a few examples of herbs being used successfully in treating the symptoms and problems associated with diabetes. None of the studies I found involved the plant *Chimaphila maculata*, demonstrating perhaps how much research has still not been done in this field and the need for more research projects like this.

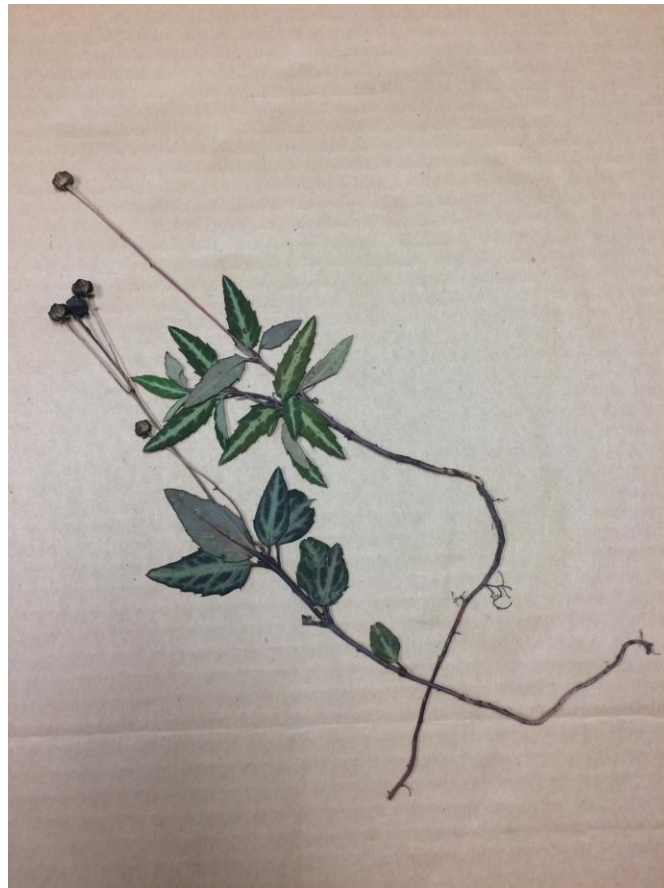


Figure 1. *Chimaphila maculata* preserved by drying in the oven.

Chimaphila maculata, which is commonly referred to as both spotted wintergreen and pipsissewa, is a plant used to treat diabetes as well as general stomach trouble, arthritis, backache, neuritis, rheumatism, bladder problems, as a diuretic, an astringent and as a pain reliever. This plant was made into teas, salves and into a wash. A member of the *Pyroloideae* subfamily of the *Ericaceae* family, *C. maculata* is a perennial with white or pink flowers and thick waxy leaves that have a white line in the center. This plant has been used as a urinary tract disinfectant, is believed to help break up kidney stones and was shown to lower blood glucose levels in animal studies (Boughman, 2004).

Materials and Methods

Twelve common bacterial strains were used in this experiment: *B. subtilis*, *C. xerosis*, *E. aerogenes*, *E. coli*, *E. faecalis*, *K. pneumonia*, *N. sicca*, *P. aureiginosa*, *P. mirabilis*, *P. vulgaris*, *S. aureus* and *S. epidermidis*. Bacterial media and agar were also needed to grow the bacteria and to make plates to test the bacteria on; the types of media used in this experiment were Luria broth, Tryptic soy and Nutrient agar. Filter discs were also needed to perform the agar diffusion assay. The filter discs were cut from a larger filter disc. Microscope slides, scalpels, microfuge tubes and a heat supply were needed to prepare the teas. The plants used in this experiment were collected from Sampson's Landing during the summer and fall of 2016 (Figure 2). These plants were kept in a minus 80 degrees Celsius freezer to preserve them for the duration of this experiment.

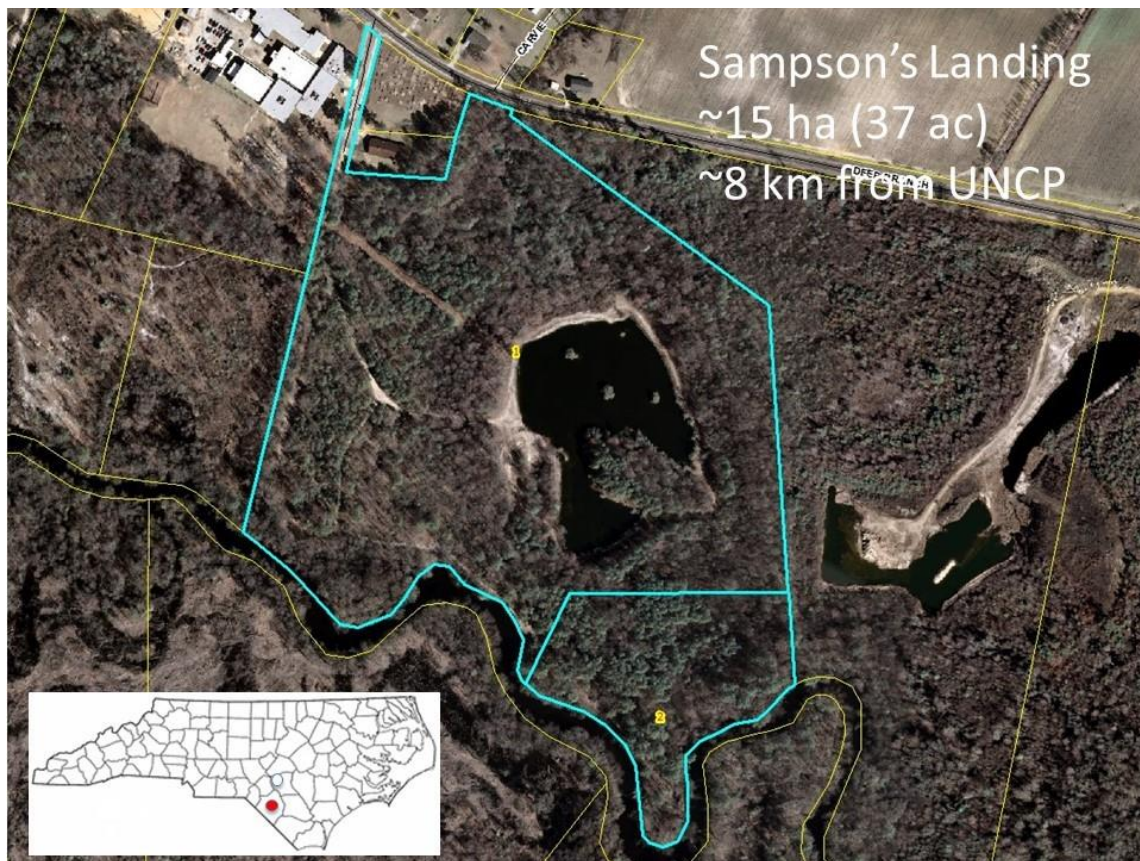


Figure 2. Aerial view of Sampson's Landing where plant collection occurred. This image was found on the GIS website for Robeson County.

To begin the experiment, media and agar had to be made. Research was done in order to see what kind of media was needed for the bacteria in the lab. The bacteria in this experiment came from glycerol stocks that were made in the lab. The media and agar powder were mixed with deoxygenated deionized water and put into the autoclave in order to be sterilized. After the media was made, the bacteria was inoculated in the media and left in the incubator overnight to grow. Agar was used to pour plates. About 20 mL of agar was poured into each plate. After the plates were poured and the bacteria was grown in liquid culture overnight, plant teas could be made. The plant teas were made using hot water extracts in order to mimic how they were used traditionally by indigenous people (Figure 3). Plant material was be separated (plant leaves were separated by the other parts of the plant) and

cut up into small pieces to allow for better extraction. The amount of plant material being used in the teas was weighed and placed into a microfuge tube. Water was added to the plant material using a micropipettor. The concentration of the tea was then calculated by taking the amount of plant material and dividing it by the amount of water added. These plants were then placed inside a heat source and were steeped for one hour at about 100° C. During the time the plants were steeping, overnight cultures of bacteria strains were swabbed onto individual plates.



Figure 3. Images of some teas made for the experiment

After the teas were steeped, single filter discs were dipped into the teas and placed onto planned sections of the plate (Figure 4, left). Isopropanol was used as the positive control (growth inhibiting) and ddH₂O was used as the negative control (no inhibition of growth). In some experiments, a plain filter disc would be used as a second positive control to ensure no filter disc contamination. A piece of the plant material such as a piece of a leaf or a stick was also directly placed on the plate to test for the antibacterial properties of the plant not steeped into a tea.

Once the plates were completely filled with filter discs and plant material, plates were placed inside an incubator at 37°C agar side up and left to grow overnight. The next day, plates were inspected for bacterial inhibition and the zone

of inhibition was measured (Figure 4, right). This experiment was repeated for accuracy.

Minimum inhibition was also tested for in this experiment. Plant teas were prepared as previously described with concentrations ranging from ten to thirty percent for the leaves and sticks of the plant. Filter disc would be dipped into the teas and placed onto plates strained with bacteria. They would be left in the incubator overnight and would be examined in the morning.



Figure 4. The two images are examples of the setup of our inhibition assay experiment. Left: idealized schematic of the different sections of the plate. Right: example of an actual plate after overnight incubation.

Results

Half of the bacterial strains used in this experiment were inhibited by teas made from *Chimaphila maculata* using agar diffusion assays (representative images shown in Figure 5). Experiments were repeated at least two times for each bacterial strain.

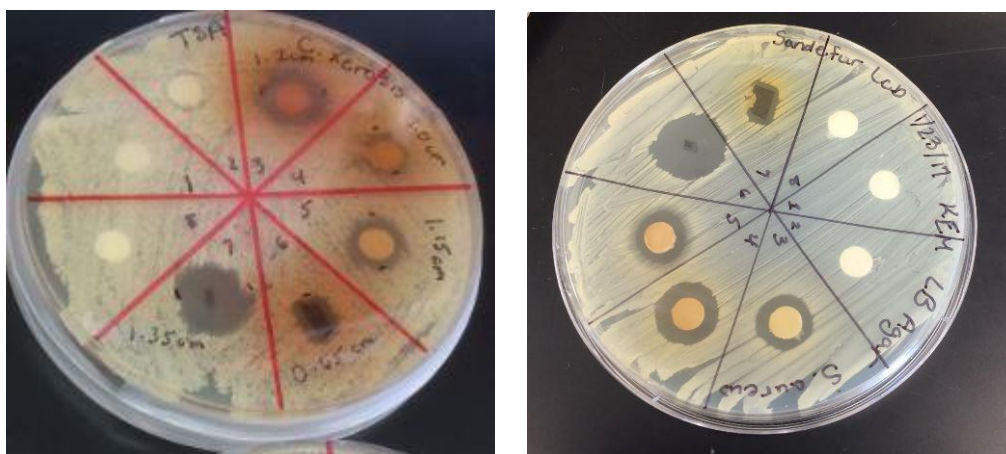


Figure 5. Representative results of agar diffusion assays. *C. xerosis* is on the left and *S. aureus* is on the right.

Bar graphs of the average zone of inhibition for growth-inhibited bacteria whose growth were generated (Figure 6). Summary results across all bacteria are located in Table 1. The largest zone of inhibition occurred after treatment of *B. subtilis* by 39% teas made from *C. maculata* leaves (Figure 6, top left). Additionally, *B. subtilis* was inhibited by teas made from both leaf or stick as well as both the leaf and stick material. There was a general increase in the zone of inhibition as the concentration of tea (both leaf or stick) increased. *C. xerosis* (Figure 6, top right) had varying zones of inhibition across the different concentration teas. A single piece of the stick had the largest zone of inhibition. *E. facecails* (Figure 6, middle left) was inhibited by two teas made from the leaves and by a piece of stick from the plant. A piece of the leaf and a stick tea with a concentration of 54% were not able to inhibit the plant. The largest inhibition of *E. facecalis* was by the leaf tea with a concentration of 57%. *P. mirabilis* (Figure 6, middle right) was only inhibited by one of the teas, the leaf tea with a concentration of 54%. Although this was the only tea that caused inhibition, the zone of inhibition was slightly larger the zone of inhibition from the positive control which was isopropanol. *P. vulgaris* (Figure 6, bottom left) had an increase in the zone of inhibition as the concentration of the teas increased. All four teas in this trial were made from the leaves although when a piece of leaf was used to test for inhibition, there was no inhibition of the bacteria. A

piece of stick from the plant was able to inhibit bacterial growth. *S. aureus* (Figure 6, bottom right) also displayed a similar pattern to *P. vulgaris* because as the concentration of the teas increased, the zone of inhibition increased. A piece of the stick from the plant had the largest zone of inhibition.

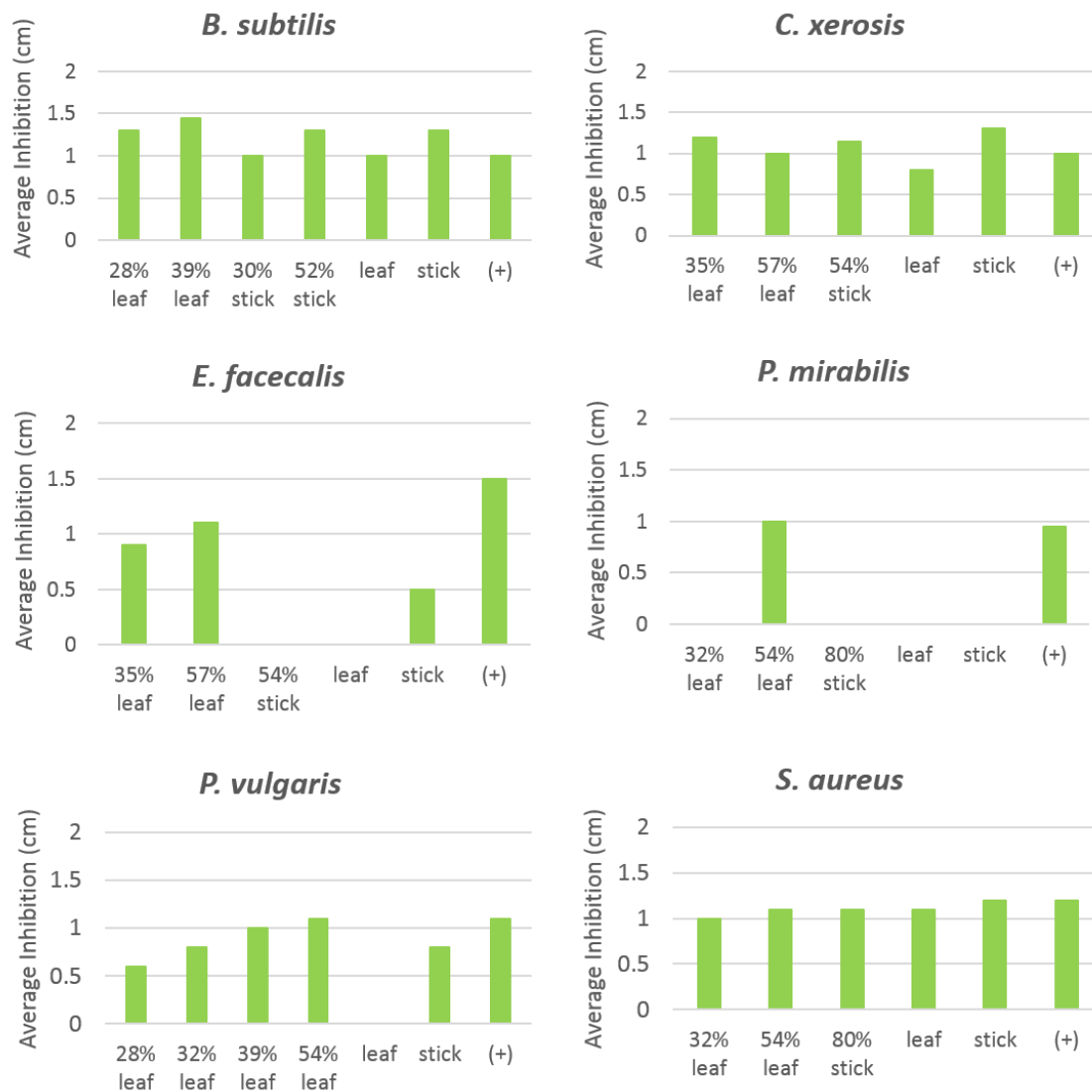


Figure 6. Bar graphs showing the average inhibition for the six strains showing inhibition. Positive control (+) was 70% isopropanol. The negative control (-), ddH₂O, is not shown to save space.

Table 1. Half (six) of bacteria tested showed growth inhibition by *C. maculata* hot water extracts

Bacteria that were inhibited	Bacteria that were not inhibited
<i>S. aureus</i> (figure 11)	<i>E. coli</i>
<i>P. vulgaris</i> (figure 10)	<i>P. aeruginosa</i>
<i>P. mirabilis</i> (figure 9)	<i>N. sicca</i>
<i>B. subtilis</i> (figure 6)	<i>K. pneumoniae</i>
<i>C. xerosis</i> (figure 7)	<i>E. aerogenes</i>
<i>E. faecalis</i> (figure 8)	<i>S. epidermidis</i>

To test for minimum inhibitory concentration, *B. subtilis* and *S. aureus* were selected because these bacteria showed the largest zones of inhibition. If there was more time available, then all bacteria that were inhibited by the teas would have been tested. Teas from the plant leaves were made with a concentration that ranged from 10% to 30%. Also teas made from the plant sticks were made with a concentration that ranged from 10% to 30%. Representative agar diffusion results are depicted in Figure 7. A minimum inhibitory concentration was not determined for *B. subtilis* suggesting minimum inhibition is below 10% for teas made from both leaves and sticks (Figure 8, blue). The minimum inhibition for *S. aureus* was determined to fall between 10% and 20% for teas made from sticks and leaves (Figure 8, green).

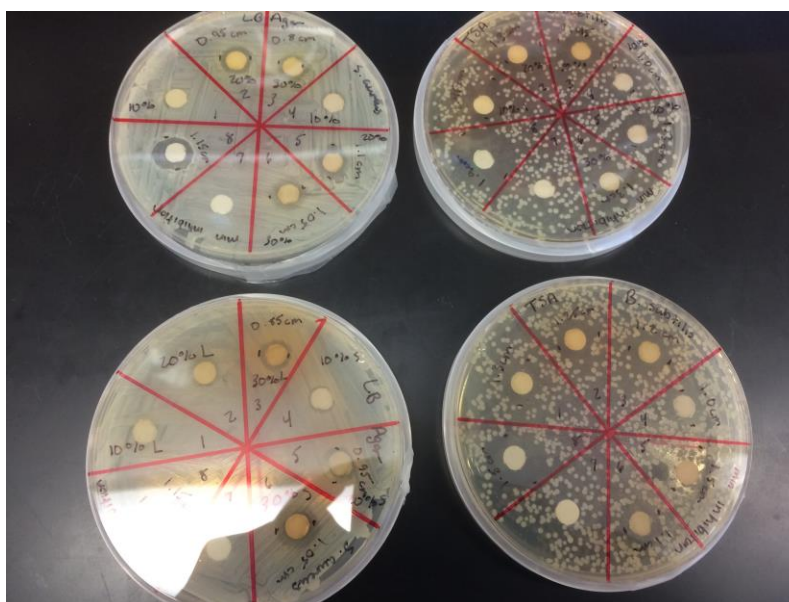


Figure 7. Representative results of agar diffusion assays on the minimum inhibition experiments. The plates on the left are *S. aureus* and the plates on the right are *B. subtilis*.

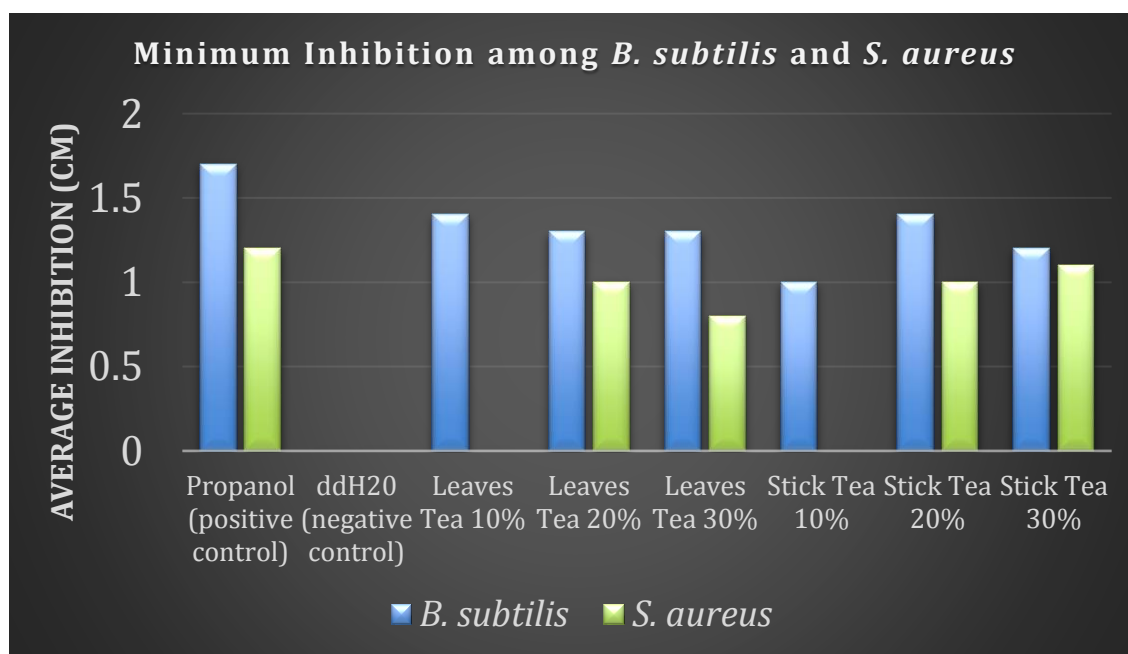


Figure 8. Minimum inhibition of growth differed between *B. subtilis* (blue) and *S. aureus* (green).

Discussion

From the results of this experience, there is evidence that *Chimaphila maculata* has some antibacterial properties. There is not a lot of available literature on this experiment so there is no scientific evidence to compare my results with. There are studies on gut bacteria related to diabetes that can be used to analyze what the results mean. One study found that there is an increase in bacteria belonging to the group *Firmicutes* in people with diabetes and that modification of these bacteria can be used to control obesity and diabetes (Ray, 2013). Four bacteria used in this experiment belonged to the phylum *Firmicutes* and out of those four, three were inhibited by *Chimaphila maculata*, suggesting this plant might be a possible positive benefit to individuals with type 2 diabetes. Certainly, further studies are necessary.

Another implication of this research that we would like to analyze is whether this plant disrupt the normal microbiome of humans. All of the bacteria used in this

experiment are part of the human biome, yet most if not all of the bacteria are not found in the gut. For example, *S. epidermidis* is most commonly found on the skin. Although *C. maculata* may be a treatment option in diabetes, we want to make sure that it does not disrupt normal microbiome of the human body because this could also cause illness. Testing the majority of bacteria of the human biome is one way to demonstrate that this plant is only effective in areas where it is needed and will not disrupt the delicate balance of the human body.

After determining which bacteria were inhibited we tried to determine if there was a pattern between bacteria that were inhibited and bacteria that were not inhibited. We identified the different family, genus, phylum of the bacteria and also determined if the bacteria were gram positive or negative (Table 2). So far no pattern has been established between bacteria that were and were not inhibited. For the future of this project, we will continue to search for a pattern. In the future we would also like to utilize human-gut-dwelling bacteria in this project to find closer evidence that this plant can be used as a treatment for diabetes.

Table 2. There are no straightforward relationships between the bacteria inhibited (black) or the bacteria not inhibited (green). The bacteria used are aerobically grown and are all common laboratory strains.

Bacteria	Phylum	Family	Genus	Gram
1. <i>B. subtilis</i>	Firmicutes	Bacillaceae	Bacillus	+
2. <i>C. xerosis</i>	Actinobacteria	Corynebacteriaceae	Corynebacterium	+
3. <i>E. aerogenes</i>	Proteobacteria	Enterobacteriaceae	Enterobacter	-
4. <i>E. coli</i>	Proteobacteria	Enterobacteriaceae	Escherichia	-
5. <i>E. faecalis</i>	Firmicutes	Enterococcaceae	Enterococcus	+
6. <i>K. pneumoniae</i>	Proteobacteria	Enterobacteriaceae	Klebsiella	-
7. <i>N. sicca</i>	Proteobacteria	Neisseriaceae	Neisseria	-
8. <i>P. aeruginosa</i>	Proteobacteria	Pseudomonadaceae	Pseudomonas	-
9. <i>P. mirabilis</i>	Proteobacteria	Enterobacteriaceae	Proteus	-
10. <i>P. vulgaris</i>	Proteobacteria	Enterobacteriaceae	Proteus	-
11. <i>S. aureus</i>	Firmicutes	Staphylococcaceae	Staphylococcus	+
12. <i>S. epidermis</i>	Firmicutes	Staphylococcaceae	Staphylococcus	+

Conclusion

This project confirmed that the plant *Chimaphila maculata* has antibacterial on some common bacterial laboratory strains. Also searching through the literature concluded that *Chimaphila maculata* may be a treatment option for diabetes because of the inhibition of bacteria belonging to the phylum *Firmicutes*. Because of the lack of literature on this plant being used to treat diabetes, there is a need for more research in this field.

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